PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/57, 9/52, 5/12, C07K 14/205, 14/28, 16/12, C12Q 1/68, G01N 33/577, 33/68

A1

(11) International Publication Number: WO 95/01445

(43) International Publication Date: 12 January 1995 (12.01.95)

(21) International Application Number: PCT/GB94/01406

(22) International Filing Date: 29 June 1994 (29.06.94)

(30) Priority Data:
9313437.7 30 June 1993 (30.06.93) GB

(71) Applicant (*for all designated States except US*): RECKITT & COLMAN PRODUCTS LIMITED [GB/GB]; One Burlington Lane, London W4 2RW (GB).

(72) Inventor; and

(75) Inventor/Applicant (*for US only*): SMITH, Andrew, William [GB/GB]; 33 Burnhill Road, Beckenham, Kent BR3 3LA (GB).

(74) Agent: BOULT WADE TENNANT; 27 Furnival Street, London EC4A 1PQ (GB).

(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LU, LV, MD, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: HELICOBACTER PYLORI HAEMAGGLUTININ/PROTEASE PROTEIN, NUCLEIC ACID ENCODING THEREFOR AND ANTIBODIES SPECIFIC THERETO

(57) Abstract

Helicobacter pylori (*H.pylori*) haemagglutinin/protease protein, nucleic acids encoding therefor and antibodies specific thereto are described and, in particular, to their use in the identification of *H.pylori* and in the diagnosis of *H.pyroli* infection. Also described are kits for the identification and diagnosis of *H.pylori* infection.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgystan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo			SE	Sweden
CH	Switzerland	KR	Republic of Korea	SI	Slovenia
CI	Côte d'Ivoire	KZ	Kazakhstan	SK	Slovakia
CM	Cameroon	LI	Liechtenstein	SN	Senegal
CN	China	LK	Sri Lanka	TD	Chad
CS	Czechoslovakia	LU	Luxembourg	TG	Togo
CZ	Czech Republic	LV	Latvia	TJ	Tajikistan
DE	Germany	MC	Monaco	TT	Trinidad and Tobago
DK	Denmark	MD	Republic of Moldova	UA	Ukraine
ES	Spain	MG	Madagascar	US	United States of America
FI	Finland	ML	Mali	UZ	Uzbekistan
FR	France	MN	Mongolia	VN	Viet Nam
GA	Gabon				

HELICOBACTER PYLORI HAEMAGGLUTININ/PROTEASE PROTEIN, NUCLEIC ACID ENCODING THEREFOR AND ANTIBODIES SPECIFIC THERETO.

The present invention relates to Helicobacter pylori (H.pylori) haemagglutinin/protease protein, nucleic acids encoding therefor and antibodies specific thereto and, in particular, to their use in the identification of H.pylori and in the diagnosis of H.pylori infection.

H.pylori (formerly Campylobacter pyloridis or C.pylori) is a spiral-shaped Gram negative microorganism which appears to live beneath the mucus layer of the stomach. Since its first isolation in 1982 H.pylori has been associated with gastric and duodenal ulcer disease and gastric cancer. H.pylori has been described as the most chronic infectious agent of man. Reviews on the state of the art include those by C.A.M. McNulty in J. Infection, 1986, 13, 107-113, C.S. Goodwin et al. in J. Clin. Pathol., 1986, 39, 353-365 and the Eurogast Study Group, Lancet, 1993, 341, 1359-1362.

The number of genes that encode proteins that are involved in the ability of H.pylori to cause disease is unknown and virulence determinants of H.pylori have so far not been identified. A number of determinants possessed by this organism have been proposed as possible pathogenic factors. For example, multiple flagella allow the microorganism to move rapidly by a corkscrew-like motion through highly viscous fluids such as the mucus layer of the gut which normally poses a barrier to bacteria en route to the gut epithelium. Also, the ability of H.pylori to produce mucinase digesting enzymes allows the organism to spread in the stomach. Microscopic studies of gastric biopsies from patients with H.pylori infection

have shown the H.pylori organisms at specific sites on the gastric epithelial cells. Biochemical studies have reported the identification of haemagglutinins that allow H.pylori to adhere to these sites.

Extracellular metalloprotease enzymes are common microbial pathogenicity factors in bacteria causing disease in mammals. Zinc metalloprotease enzymes are known to have rapid substrate turnover and broad substrate profiles. Reviews on the state of the art are by Frausto da Silva J.J.R and Williams R.J.P in, The Biological Chemistry of the Elements, 1993, Clarendon Press, Oxford, Chapter 11, and Vallee, B.L. and Auld D.S., Biochemistry, 29, 5647-5659.

The zinc metalloprotease enzyme of Pseudomonas aeruginosa (also known as the elastase enzyme) has been shown to be important in the lung tissue-destructive processes caused by this organism in cystic fibrosis patients, (Bever R.A. and Iglewski B.H., J.Bacteriol., 1988, 170, 4309-4314). Similarly, the zinc metalloprotease enzyme of Vibrio cholerae (V.cholerae) (also known as the mucinase enzyme or haemagglutinin/protease (HAP) enzyme) has been shown to be important in the attachment and detachment of these organisms during the disease cholera. References on the state of the art include: Hase C.C. and Finkelstein R.A., J. Bacteriol., 1991, 173, 3311-3317; and Finkelstein R.A. et al., Infect.Immunol. 1992, 60 472-478.

We have now surprisingly found that a virulance gene almost identical to the V.cholerae hap gene, which we have termed the H.pylori hap gene, is present in the H.pylori genome. The detection of the H.pylori hap nucleic acid sequence by polymerase chain reaction (PCR) or other hybridization methods, the detection of H.pylori HAP protein epitopes by antibody detection methods, or the detection of antibodies to the

H.pylori HAP protein by, for example ELISA, have utility in the diagnosis of H.pylori mediated gastroduodenal disorders in mammals.

Prior art in the diagnosis of H.pylori mediated gastric diseases include Campylobacter DNA probes capable of hybridizing H.pylori RNA (EP-A-0350205). H.pylori oligonucleotides specific for the H.pylori urease gene sequences are disclosed in WO 91/09049. Serological detection and diagnosis of H.pylori infection by serological immunoassays and detection of H.pylori antigens and antigenic fragments are disclosed in WO 89/08843, WO 89/09497 and EP-A-0329570.

It would be highly desirable to have a reliable means of detecting H.pylori DNA, RNA or antibodies directed against specific H.pylori proteins or H.pylori proteins themselves in clinical samples from a patient (for example in the gastric mucosa, saliva, faecal samples plasma or serum), as means of early diagnosis of gastritis, gastric or peptic ulcerations, or of gastric cancer.

Accordingly, the present invention provides a nucleic acid sequence encoding the H.pylori HAP protein or a fragment thereof comprising all or part of the nucleic acid sequence of Fig. 1.

The H.pylori hap gene of the present invention has been sequenced, giving rise to the possibility of constructing selected oligonucleotide or protein epitope sequences specific to H.pylori. The sequenced H.pylori hap gene has been found to be over 99% similar to the V.cholerae hap gene in the coding region. The nucleotide sequence of the cloned 1.5 kb H.pylori hap gene fragment showing the 1 kb of coding sequence and about 500 bp of 3′ flanking sequence is shown in Fig. 1.

Preferably the nucleic acid sequence encodes at least one antigenic determinant of H.pylori HAP

protein. Preferably the nucleic acid sequence comprises the sequences of bases numbered from 1 to 936 of Fig. 1. Also provided by the present invention is a nucleic acid sequence which is complementary to the H.pylori hap nucleic acid sequence as defined above. The nucleic acid sequence may comprise genomic DNA, complementary DNA (cDNA), synthetic DNA or recombinant DNA or RNA.

The nucleic acid sequence may comprise an oligonucleotide of from 15 to 50 nucleotides preferably from 18 to 50 nucleotides which has specific binding affinity for a portion of the nucleic acid sequence as shown in Fig. 1, or a nucleic acid sequence complementary thereto. The oligonucleotide may also be from 15 to 30 nucleotides, preferably from 15 to 25 nucleotides. Preferably the oligonucleotide comprises a sequence of at least 15 or more nucleotides and includes the nucleotides numbered 16 to 18 (inclusive), 220 to 222 (inclusive) or 43 to 45 (inclusive) as shown in Fig. 1.

Preferably the oligonucleotides are any of the following sequences:

5'- GCACAGGCAACAGGAACC-3'; or
5'- AACGAGGCCTGAATTCTGC-3'; or
5'- ATAACGTAGACCACCGGAGG-3'; or
5'- TCCGGTGGTATTAACGAAGC-3'.

The oligonucleotides may comprise DNA or RNA sequences.

The oligonucleotides within the scope of the present invention include both single- and double-stranded versions, it being understood that in any hybridization procedures such double stranded probes will require denaturing to provide the probes in single-stranded form.

The present invention further provides vectors which comprise any nucleic acid sequence as hereinbefore defined. The present invention specifically contemplates the provision of any vector system known in the art including cloning vectors such as pUC18 and pUC19 as well as expression vectors such as pASK60-Strep. Further provided by the present invention is a host cell transformed with one or more such vectors. The present invention also provides a process for the production of DNA sequences as hereinbefore defined comprising culturing a host cell which has been transformed with one or more vectors comprising the DNA sequence and isolating the DNA sequence therefrom. Such a process is carried out according to conditions and procedures well known in the art.

Further provided by the present invention is H.pylori HAP protein or a fragment thereof comprising all or a part of the amino acid sequence of Fig. 1. Preferably the fragment of the HAP protein is an antigenic determinant of H.pylori HAP protein. Preferably the fragment of the HAP protein is encoded by the sequence of bases numbered from 1 to 936 of Fig. 1.

The H.pylori protein or fragment thereof within the scope by this invention include the H.pylori HAP protein itself, being purified from H.pylori or being produced as a recombinant protein in, for example Escherichia coli or Bacillus subtilis, it being understood that the subsequent diagnostic procedures such as the detection of the H.pylori protein in the gastric mucosa or other secretions or products of the gastrointestinal tract are within the scope of this invention.

The present invention also provides a process for the production of H.pylori HAP protein or a

fragment thereof as hereinbefore described comprising culturing a host cell transformed with an expression vector as hereinbefore defined and isolating the protein or protein fragment produced therefrom.

It will be understood that in accordance with the present invention a defined nucleic acid sequence includes not only the identical nucleic acid sequence but also any minor base variations from the natural nucleic acid sequence including, in particular, substitutions in bases which result in a synonym codon (a different codon specifying the same amino acid). Furthermore a defined protein, polypeptide or amino acid sequence includes not only the identical amino acid sequence but also minor amino acid variations from the natural amino acid sequence including, in particular, conservative amino acid replacements (a replacement by an amino acid that is related in its side chains). Also included are amino acid sequences which vary from the natural amino acid but result in a polypeptide which is immunologically identical with the polypeptide encoded by the naturally occurring sequence. This includes a correspondingly altered encoding nucleic acid sequence.

The present invention also provides polyclonal and monoclonal antibodies which recognize an antigenic determinant of H.pylori HAP protein or a fragment thereof. The H.pylori specific antibodies covered by this invention include both polyclonal or monoclonal antibodies directed against the H.pylori HAP protein or a fragment thereof, it being understood that the subsequent diagnostic procedures such as the detection of antibodies against H.pylori in the gastric mucosa or other secretions or products of the gastrointestinal tract are within the scope of this invention.

The polyclonal and monoclonal antibodies are

produced by standard techniques known in the art, for example monoclonal antibodies are produced by the techniques as described in Kohler F. & Milstein C., (1975), Nature, 256, 495-497.

In accordance with the present invention, the specific H.pylori HAP protein is useful in raising antibodies to the H.pylori HAP protein in experimental mammals and these specific anti-H.pylori HAP antibodies are useful in detecting the H.pylori HAP protein in clinical samples from a patient. The H.pylori HAP protein is also useful in ELISA or Western blot analysis, and can be purified from H.pylori organisms or produced by recombinant DNA technology, the procedures for which are all well recognised and well within the capabilities of the person skilled in the art.

Further provided by the present invention are hybridomas capable of producing monoclonal antibodies which recognize an antigenic determinant of H.pylori HAP protein, or a fragment thereof.

The present invention also provides a process for the amplification of a nucleic acid sequence as hereinbefore described by polymerase chain reaction (PCR) or equivalent technique known in the art such as transcription-aided amplification system (TAS). Preferably the PCR process is effected using the oligonucleotide pairs:

i) 5'- GCACAGGCAACAGGAACC-3';
and
5'- AACGAGGCCTGAATTCTGC-3'; or

ii) 5'- ATAACGTAGACCACCGGAGG-3';
and
5'- TCCGGTGGTATTAACGAAGC-3'

The present invention also provides nucleic acid

probes comprising a nucleic acid sequence or part thereof as shown in Fig. 1. Also provided is a process for the identification of H.pylori nucleic acid comprising contacting a sample to be tested with a nucleic acid probe as hereinbefore described, under appropriate conditions known in the art, and detecting any hybridization of H.pylori nucleic acid sequence or sequences with the probe.

The selection of a particular probe, or pair of probes, will depend upon a number of factors, well understood in the art, and including amongst others the stringency requirements, i.e. the ability or otherwise of the probe to tolerate mismatching with the complementary sequence in the target DNA. Obviously the longer the probe the better the ability to withstand local mismatch without adversely affecting the hybridization of the probe to the target DNA. The factors affecting the choice of probe are well recognised and well within the capabilities of the person skilled in the art.

Also provided is a process for the identification of H.pylori nucleic acid comprising amplifying, in a sample to be tested, any H.pylori nucleic acid sequence as shown in Fig. 1 by PCR or equivalent technique and detecting the amplified nucleic acid sequences.

Although PCR amplification is the preferred method of H.pylori detection using H.pylori specific nucleotides of this invention, other detection procedures are available and are well known in the art. To this end the H.pylori specific oligonucleotides or nucleic acid probes of this invention may be provided with a variety of different labels such as radioactive, fluorescent or enzyme labels, all permitting the detection of any hybridized nucleotide bound to the unidentified nucleic acid

sample under investigation.

Further provided is a method for the identification of H.pylori HAP protein antigenic determinants comprising contacting a sample to be tested with an antibody according to the present invention and detecting the presence of an antibody-antigen complex. Synonomously, there is also provided a method for the identification of H.pylori infection comprising contacting a sample to be tested with an H.pylori HAP protein or fragment thereof as hereinbefore described, or V.cholerae HAP protein or fragment thereof, and detecting the presence of an antigen-antibody complex. The sample to be tested will generally comprise dental plaque, saliva, gastric juices, or faeces or may comprise a sample of the gastric mucosa. As described above for the H.pylori hap nucleic acid sequences of the present invention the amino acid sequences, polypeptides, protein and antibodies may be provided with a variety of different labels such as fluoroescent, radioactive or enzymic labels, all permitting the detection of any amino acid sequence or antibody to the antibody or amino acid sequence sample under investigation, respectively.

H.pylori HAP antigens (the HAP protein or a fragment thereof) or V.cholerae HAP antigens, can be used in immunoassays to detect patients whom exhibit cross-reacting antibodies. Conversely antibodies can be used in immunoassays to detect patients whom exhibit cross-reacting H.pylori HAP antigens. Correlation can thus be made with H.pylori infection-associated gastroduodenal disease. The immunoassays contemplated by the present invention comprise diagnostic methods known in the art. The immunoassays may be based on direct antigen-antibody reactions, competition, single or double sandwich assays and include amplification systems such as those

utilizing biotin and avidin.

The assays may comprise components attached to solid supports such as immunodiagnostic plates or glass beads or may involve immunoprecipitation. The immunoassays generally comprise use of a labelled antibody/antigen wherein the label may comprise fluorescent, chemiluminescent, radioactive or dye molecules.

Further, the present invention provides the use of the H.pylori hap nucleic acid, HAP amino acid, polypeptides, protein sequences and antibodies or V.cholerae hap nucleic acid or HAP protein or a fragment thereof for the manufacture of materials and kits for the diagnosis of gastric disorders associated with H.pylori. Accordingly kits are provided for by the present invention which comprise one or more of: a nucleic acid sequence encoding H.pylori HAP protein or a fragment thereof comprising all or part of the nucleic acid sequence of Fig. 1; H.pylori HAP protein or a fragment thereof comprising all or part of the amino acid sequence of Fig. 1; or V.cholerae HAP protein or a fragment thereof; or a polyclonal or monoclonal antibody which recognizes an antigenic determinant of the H.pylori HAP protein or a fragment thereof. There is also provided a kit for the identification of H.pylori using the polymerase chain reaction or equivalent technique comprising at least one of the pairs of oligonucleotides as hereinbefore described.

Kits according to the present invention may include appropriately labelled reagents, additional reagents and materials such as buffer solutions, means for detecting results of the assay and assay instructions. The kit components may be packaged in a suitable kit-container.

The present invention also provides the use of

the following in the diagnosis of H.pylori infection: a nucleic acid sequence encoding H.pylori HAP protein or a fragment thereof comprising all or part of the nucleic acid sequence of Fig. 1; H.pylori HAP protein or a fragment thereof comprising all or part of the amino acid sequence of Fig. 1; or V.cholerae HAP protein or a fragment thereof; or a polyclonal or monoclonal antibody which recognizes an antigenic determinant of H.pylori HAP protein or a fragment thereof.

Thus, the use of the H.pylori hap nucleic acid sequence or fragment thereof or protein sequence or fragment thereof or antibodies directed against the H.pylori HAP protein covered by this invention include the manufacture of a kit or other materials for use in the diagnosis of gastric disorders associated with H.pylori. The H.pylori hap nucleic acid sequence or fragment thereof or protein sequences or fragment thereof or anti-H.pylori HAP antibodies may be combined with one or more other nucleic acid sequence or fragment thereof or protein sequence or fragment thereof or antibodies used in the diagnosis of gastric disorders associated with H.pylori.

The present invention is further described with reference to the following drawings in which:

Fig. 1 is a nucleotide sequence of the cloned 1.5kb H.pylori hap gene fragment from H.pylori NCTC 11638 showing about 1kb of coding sequence and about 500bp of 3′ flanking sequence. The sequence of this region of the H.pylori hap gene has been submitted to the EMBL Nucleotide Sequence Data Library under the accession number Z27239.

Fig. 2 is a map of the 1.5kb fragment of the H.pylori hap gene in the plasmid pUC19. The H.pylori hap gene fragment is situated between the two *Bam*HI sites.

In Fig. 1. the numbering is from the first adenosine base (A) of the EcoRI site. The PCR primers are shown as dotted overlined arrows at positions 206-225 (HpHAP3), and 762-780 (HpHAP4). The three base in-frame deletion (marked Δ H) from the V.cholerae hap gene is at position 222, and the region identical to the V.cholerae hap gene extends from position 1 beyond the coding region (double underlined) except for a single addition at position 798 and a single deletion (of a T) at position 843. The stop codon is indicated by ***.

Within the identified H.pylori hap nucleic acid sequence two regions have been identified as comprising coding regions for the active site component of the protein. The two regions are around nucleic acids numbered 16-18 (inclusive) encoding for tyrosine and 220-222 (inclusive) encoding for histidine. The region around nucleic acids numbered 43-45 (inclusive) encoding for glutamic acid has been identified as a putative zinc binding encoding region.

Within the sequence in Fig. 1 certain regions of the gene containing sequences have been identified that do not cross-hybridize with Campylobacter DNA. These include the nucleotides numbered as 1-174 (inclusive).

The present invention will now be described in more detail with reference to the following examples:

Example 1

Growth of H.pylori strains and extraction of DNA and proteins

H.pylori strains NCTC (National Collection of Type Cultures, London) 11637, 11638, 11916 and HP 34 (clinical isolate from a biopsy taken during endoscopy

of a patient at Queen's Medical Centre, The University Hospital, Nottingham) were grown on Columbia blood agar with 5% horse blood (Oxoid) as a lawn for DNA extraction, for 2 days under microaerophilic conditions (Campypak, BBL) at 37°C. The resulting growth was harvested from four plates and was first Gram stained to identify the characteristic morphology. The cells were washed in 1ml of lysis buffer (50mM EDTA, 100mM NaCl), and resuspended in 400μl of lysis buffer to which 30μl of lysis buffer containing 20% N-lauroylsarcosine (Sigma) was added. After five minutes incubation at room temperature, the suspension was repeatedly extracted with phenol saturated with TE (10mM Tris-HCI, pH 8, 1mM EDTA)buffer until no interface was evident. The nucleic acids were then ethanol precipitated overnight, collected by centrifugation, washed in 70% ethanol and the pellet air dried for 10 minutes. The DNA was then treated with proteinase K and purified using a Qiagen minicolumn according to the manufacturer's instructions. H.pylori strain NCTC 11638 was grown in liquid culture by adding one harvested plate of culture to 100ml of Brucella broth (Difco) containing 2% β-cyclodextrin (Sigma) and 0.2ml of reconstituted H.pylori selective supplement (Oxoid) in a 500 ml conical flask. The flask was incubated in an anaerobic jar with gentle shaking (100 r.p.m) for 3 days under microaerophilic conditions (Campypak, BBL) at 37°C. The H.pylori cells were collected by centrifugation and the proteins in the supernatant precipitated as previously described in Milton D.L., Norqvist, A., and Wolf Watz, H., (1992), J. Bacteriol., 174,7235-7244. Cellular proteins were extracted by resuspending two plates of growth in 1.5 ml of protein-extraction buffer (10mM Tris pH 7.5, 1mM $MgCl_2$, 0.15mM EDTA, 1mM DTT, 1mM PMSF, 2μgml^{-1}

pepstatin A (Sigma) 0.5μgml^{-1} leupeptin (Sigma)), adding 200μl of 10% SDS and boiling for 5 minutes. The suspension was then cooled on ice for 5 minutes and the supernatant collected by centrifugation at 12500 r.p.m. for five minutes. The protein concentration was determined according to Bradford, M., (1976), Anal Biochem, 72,248-252, (Bio-Rad kit).

Example 2

Identification of a H.pylori protease enzyme

40μg of total cell and supernatant proteins from H.pylori NCTC 11638 and a clinical isolate of P.aeruginosa were separated on vertical minigels (Hoeffer Scientific), comprising a 5% acrylamide stacking gel and a 13% resolving gel, according to the procedure of Laemmli, UK., (1970), Nature, 227, 680-685. Electrophoresis was performed at 20mA. Half of the gel was stained directly and the other half was incubated before staining to reveal protease activity. The gel portion that was stained directly was placed in a solution of 0.1% Coomassie brilliant blue R-250 in destain solution (40% methanol, 10% acetic acid, 50% water) for 1h, and then placed in several changes of destain solution. The unstained gel was incubated, and the protease activity of this gel was detected by the procedures as described in Milton et al., 1992, J.Bacteriol., 174,7235-7244, except that the gel was finally stained with Coomassie brilliant blue R-250 as above rather than amido black. Protease activity was identified in the gel by cleared bands (digestion of the gelatin and other proteins). Protease activity was clearly present in the H.pylori cell, supernatant and P.aeruginosa tracks. In the H.pylori tracks numerous proteolytic

bands were present. An overloaded gel (100μg of total H.pylori protein) run according to the above procedure showed a clear protease band at about 35kDa.

The electrophoretic separation was transferred on to a nitrocellulose membrane by semi-dry blotting using transfer buffer (40mM Tris pH8, 30mM glycine, 20% methanol, 1.3mM SDS) and an ATTO AE-6675 Horizblot transfer unit (Genetic Research International) according to the manufacturer's instructions. The nitrocellulose membrane was then dried and placed in blocking solution (1% bovine serum albumin in wash buffer (10mM Tris pH 7.5, 100mM NaCl,0.1% Tween 20)) for 1 hour at room temperature with constant rocking. The membrane was probed with either rabbit anti-P.aeruginosa elastase, Bever and Iglewski., 1988, J. Bacteriol., 170:4309-4314, or pooled human sera absorbed with E.coli according to techniques well known in the art. The primary antibodies were added to the blocking buffer at 1:1000 and incubation was continued for 1 to 4 hours. The membrane was then briefly washed twice with wash buffer, once for 15 minutes and once for 5 minutes with rocking. The HRP-labelled antibody (either anti-rabbit or anti-human) was added to the membrane at a concentration of 1:1000 in wash buffer and incubated for 1 hour as above. The membrane was then washed once for 15 minutes, and four times for 5 minutes with wash buffer as above. The membrane was then developed using ECL substrate reagents (Amersham) and exposed to Fuji RX X-ray film and developed according to the manufacturer's instructions. Re-probing of blots was performed after stripping by incubating in 20mM glycine pH 2.5, 0.055% Tween 20 overnight at room temperature with continuous shaking.

After probing with HRP labelled anti-rabbit antibodies, a strong band was visible in the

P.aeruginosa track and a band of similar molecular weight to the P.aeruginosa elastase was seen in the H.pylori cellular protein extracts. After probing with the pooled sera from five patients with high-titre antibodies against H.pylori, a large number of bands were observed including a strong response to the band (35kDa) previously identified by the anti-P.aeruginosa elastase antibody. After probing with pooled sera from five patients not infected with H.pylori no bands were visible. Thus, a protein of similar size and immuniological reactivity to the P.aeruginosa elastase protein was shown to be present in cellular and supernatant protein extracts of H.pylori NCTC 11638.

Example 3

Cloning of the H.pylori hap gene

5μg of *Hind*III and *Bam*HI digested H.pylori NCTC 11638 genomic DNA were separated and blotted onto Hybond N (Amersham) as described in Smith et al. (1992), Gene, 114:211-216. The genomic blots were probed overnight with 200ng of a 3.2kb *Hind*III fragment of V.cholerae hap gene DNA and 5ng of *Hind*III - digested phage lambda DNA directly labelled with HRP (Amersham ECL kit), washed, developed and exposed to Fuji RX X-ray film according to the manufacturer's instructions. A 4kb *Hind*III and a 1.5kb *Bam*HI fragment of H.pylori DNA hybridized strongly to the V.cholerae hap gene probe.

Both the 4kb (*Hind*III) and the 1.5kb (*Bam*HI) fragments were cloned from H.pylori NCTC 11638 genomic DNA by the following method:

Two 10μg portions genomic DNA were digested with either *Bam*HI or *Hind*III and size separated on

a 0.5 x TBE minigel. The 4kb *Hind*III fragment and the 1.5kb *Bam*HI fragment were extracted from the gel and ligated separately into either *Hind*III or *Bam*HI - digested (respectively) and calf alkaline phosphatased pUC18, pUC19 (Fig. 2) and pAT153 as described in Smith,A.W., (1990), Ph.D. Thesis, University of Nottingham, Nottingham, United Kingdom. The resultant plasmids were transformed into E.coli strain DH5α and plated out onto Lbroth agar plates containing 100mg/l ampillicin and incubated overnight at 37°C. The desired recombinants were indentified by colony lifts onto Hybond N and screened by colony hybridization using the 3.2kb *Hind*III V.cholerae (ECL) probe prepared above according to the manufacturer's instructions. The 1.5kb *Bam*HI fragment was sequenced in both directions using custom made, universal and reverse sequencing primers and Sequenase Version 2 (United States Biochemical Corporation) according to the manufacturer's instructions.

Sequencing of the 1.5kb fragment did not reveal a start codon but did reveal a region of about 1kb that was over 99% identical to the V.cholerae hap gene sequence with only three base in-frame deletion of a histidine residue in the coding region (ΔH in Fig. 1). The sequences diverge completely about 50bp downstream of the stop codon, with the other bases differing before the complete divergence. The cloned sequence is then quite different from the 3'flanking region of the V.cholerae hap locus. Such coding sequence conservation is highly unusual and difficult to explain either by a common precursor gene or by intrageneric gene transfer. The %G+C content for H.pylori is 34-37% while for V.cholerae it is 46-48%, the subsequent difference in codon usage between the two genera should have allowed the DNA sequences to diverge

even if the amino acid sequences were still conserved.

Example 4

PCR of H.pylori genomic DNA

50ng of H.pylori genomic DNA from the four strains NCTC 11638, NCTC 11637, NCTC 11916 and HP34 were amplified using DynaZyme (Flowgen) thermastable polymerase according to the manufacturer's instructions. The primers used were either specific to the H.pylori hap gene 5'-GCACAGGCAACAGGAACC-3' and 5'-AACGAGGCCTGAATTCTGC-3' or those previously published to amplify a 411 bp fragment in the H.pylori ureA gene (Clayton et al, 1992 J.Clin.Microbiol 30,192-200 and Lopez et al, 1993, Mol.Cell Probes, 7,439-446.

The reaction cycle profiles were as follows, one cycle at 95°C for 5 min; thirty cycles at 94°C for 30 sec, either 52°C (hap) or 48°C (ureA) for 1 min, 72°C for 1 min. 30 secs; and one cycle at 72°C for 5 min. The PCR products were separated on 1.5% agarose Tris acetate gels, stained with ethidium bromide, blotted and hybridized as described in Example 2.

All four stains gave the 575 bp product predicted from the cloned hap gene sequence, although polymorphisms similar to those seen in the Southern blot analysis of strain HP34 were evident. Similarly, all four strains gave the 411 bp product from the ureA gene thereby confirming the origin of the genomic DNA. Genomic DNA from the nine Helicobacter species H.acinonyx, H.felis, H.fennelliae, H.canis, H.muridarum, H.nemestrinae, H.mustelae, H.cinaedi and H.pylori NCTC 11638 was amplified using the HAP gene primers and all nine gave a 575 bp fragment. Some polymorphisms were observed noticeably with H.acinonyx, H.felis, H.canis and H.nemestrinae, which were also evident from Southern

analysis of genomic DNA from these species. This separation was blotted and probed with the V.cholerae hap gene and of the PCR products produced from H.pylori genomic DNA only the 575 bp hap fragment, and not the 411 bp (ureA) fragment, hybridized. The 575 bp PCR products from all nine Helicobacter species were shown to hybridize with the V.cholerae hap gene probe.

Due to the deletion between the H.pylori hap gene and the V.cholerae hap gene, PCR using non proof reading enzymes would result in no amplification of any homologous V.cholerae hap gene sequence present.

CLAIMS

1. A nucleic acid sequence encoding H.pylori HAP protein or a fragment thereof comprising all or a part of the nucleic acid sequence of Fig. 1.

2. A nucleic acid sequence as claimed in claim 1 which encodes an antigenic determinant of H.pylori HAP protein.

3. A nucleic acid sequence as claimed in claim 1 comprising the sequence of bases numbered from 1 to 936 of Fig. 1.

4. A nucleic acid sequence which is complementary to a nucleic acid sequence of any one of claims 1 to 3.

5. A nucleic acid sequence as claimed in any one of claims 1 to 4 which is genomic DNA, cDNA, synthetic DNA or recombinant DNA.

6. An oligonucleotide of from 18 to 50 nucleotides which has a specific binding affinity for a nucleic acid sequence as claimed in any one of claims 1 to 5.

7. An oligonucleotide as claimed in claim 6 having the sequence:

5'- GCACAGGCAACAGGAACC-3'; or
5'- AACGAGGCCTGAATTCTGC-3'; or
5'- ATAACGTAGACCACCGGAGG-3'; or
5'- TCCGGTGGTATTAACGAAGC-3'

8. A vector comprising a recombinant nucleic acid sequence as claimed in claim 5.

9. A vector as claimed in claim 8 which is pUC18 or pUC19.

10. A vector as claimed in claim 8 which is an expression vector.

11. A vector as claimed in claim 10 which is the expression vector pASK60-Strep.

12. A host cell transformed with a vector as claimed in any one of claims 8 to 11.

13. A process for the production of a recombinant nucleic acid sequence as claimed in claim 5 comprising culturing a host cell as claimed in claim 12 and isolating the nucleic acid sequence therefrom.

14. H.pylori HAP protein or a fragment thereof comprising all or a part of the amino acid sequence of Fig. 1.

15. H.pylori HAP protein or a fragment thereof as claimed in claim 14 expressed from a vector as claimed in claim 10 or claim 11.

16. H.pylori HAP protein fragment as claimed in claim 14 comprising the amino acid sequence encoded by the sequence of bases numbered from 1 to 936 of Fig. 1.

17. A process for the production of H.pylori HAP protein or fragment thereof as claimed in claim 14 comprising culturing a host cell as claimed in claim 12 and isolating the protein or protein fragment produced therefrom.

18. A polyclonal antibody which recognizes an

antigenic determinant of H.pylori HAP protein or a fragment thereof.

19. A monoclonal antibody which recognizes an antigenic determinant of H.pylori HAP protein or a fragment thereof.

20. A hybridoma capable of producing a monoclonal antibody as claimed in claim 19.

21. A process for the amplification of a nucleic acid sequence as claimed in any one of claims 1 to 4 by polymerase chain reaction or an equivalent technique wherein the polymerase chain reaction is effected by using the oligonucleotide pairs:

i) 5'-GCACAGGCAACAGGAACC-3'
and
5'-AACGAGGCCTGAATTCTGC-3'; or

ii) 5'-ATAACGTAGACCACCGGAGG-3'
and
5'-TCCGGTGGTATTAACGAAGC-3'.

22. A nucleic acid probe comprising a nucleic acid sequence or a fragment thereof as claimed in any one of claims 1 to 5, or an oligonucleotide as claimed in claim 6 or claim 7.

23. A method for the identification of H.pylori nucleic acid comprising contacting a sample to be tested with a nucleic acid probe as claimed in claim 22 under appropriate conditions and detecting any hybridization of H.pylori nucleic acid sequence or sequences with the probe.

24. A method for the identification of H.pylori

nucleic acid comprising amplifying, in a sample to be tested, H.pylori nucleic acid sequence or sequences as claimed in any one of claims 1 to 5 by polymerase chain reaction or an equivalent technique and detecting the amplified nucleic acid sequence or sequences.

25. A method for the identification of H.pylori HAP protein antigenic determinants comprising contacting a sample to be tested with an antibody as claimed in claim 18 or claim 19 and detecting the presence of an antibody-antigen complex.

26. A method for the identification of H.pylori infection by detecting the presence of antibodies to H.pylori HAP protein comprising contacting a sample to be tested with an H.pylori HAP protein or fragment thereof as claimed in claim 14, or V.cholerae HAP protein or a fragment thereof, and detecting the presence of an antigen-antibody complex.

27. A kit for the identification of H.pylori nucleic acid sequence comprising a nucleic acid sequence as claimed in any one of claims 1 to 5.

28. A kit for the identification of H.pylori hap gene nucleic acid sequence using polymerase chain reaction or an equivalent technique comprising a pair of oligonucleotide primers.

29. A kit for the diagnosis of H.pylori infection by the presence of anti-H.pylori HAP protein antibodies comprising H.pylori HAP protein, or a fragment thereof as claimed in claim 14, or V.cholerae HAP protein or a fragment thereof

30. A kit for the identification of H.pylori HAP protein antigenic determinants comprising a polyclonal or monoclonal antibody as claimed in claim 18 or claim 19.

31. Use of a nucleic acid sequence as claimed in any one of claims 1 to 7 in the diagnosis of H.pylori infection.

32. Use of H.pylori HAP protein or a fragment thereof as claimed in claim 14 or V.cholerae HAP protein or a fragment thereof, in the diagnosis of H.pylori infection.

33. Use of a polyclonal or monoclonal antibody as claimed in claim 18 or claim 19 in the diagnosis of H.pylori infection.

FIG. 1.

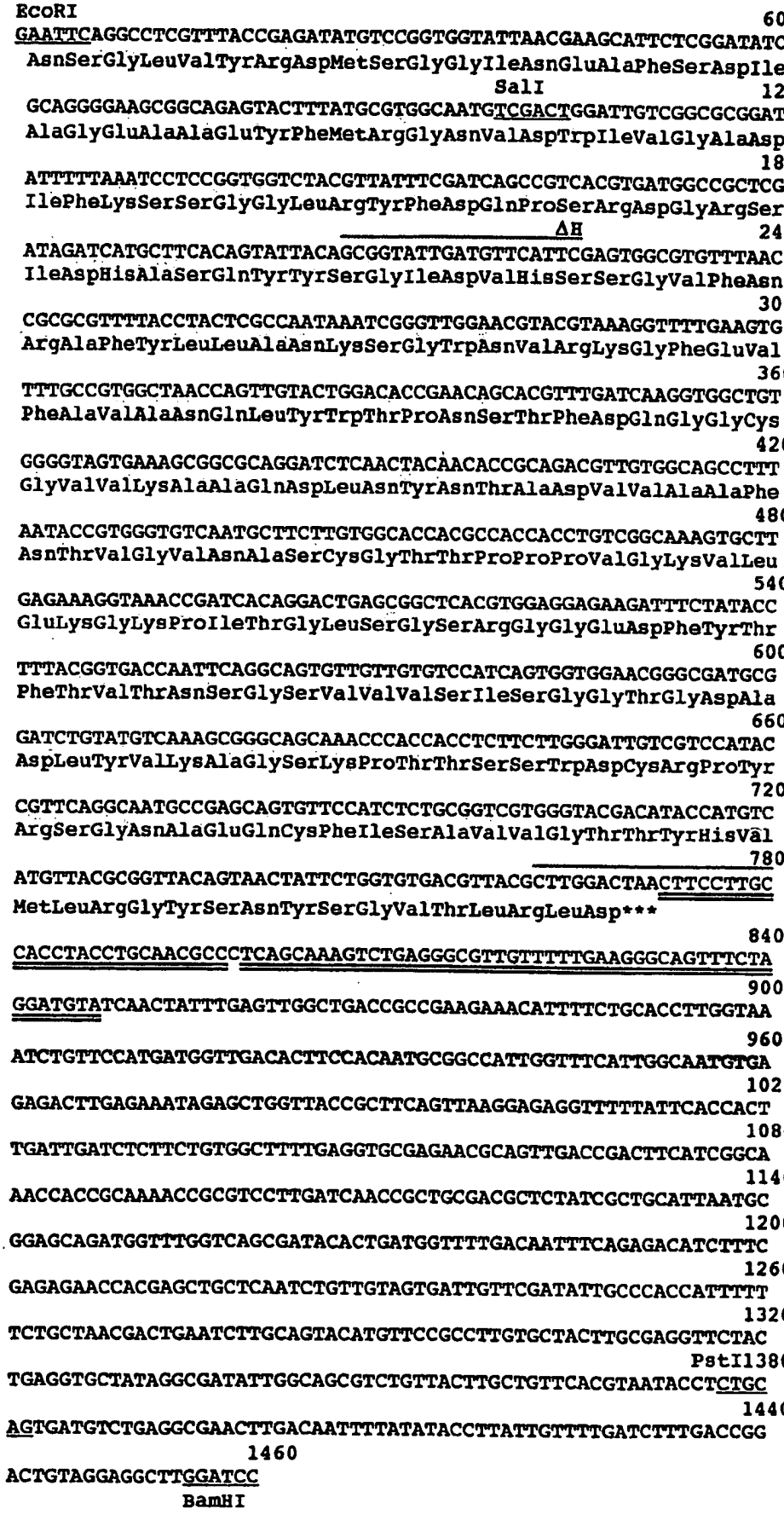
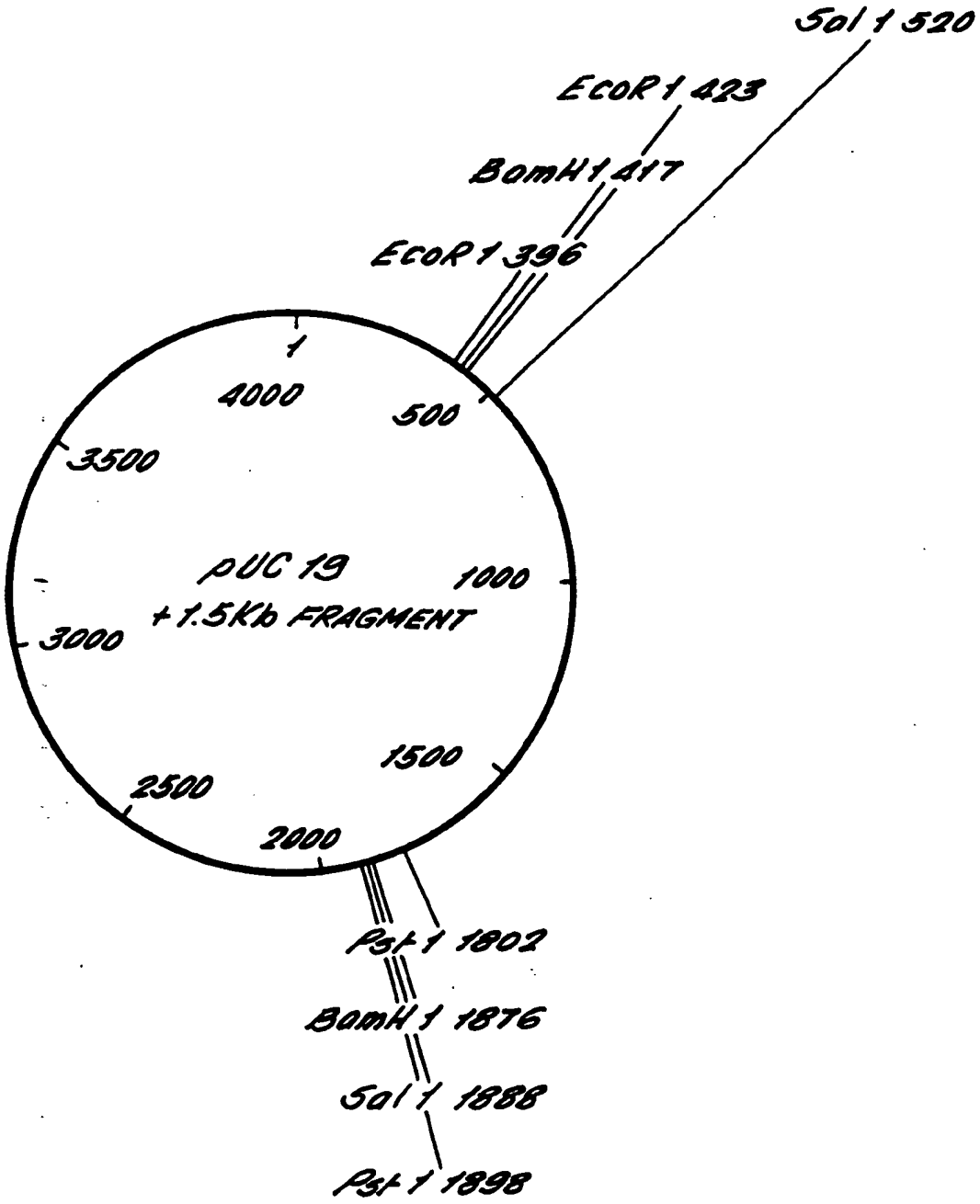

EcoRI
GAATTCAGGCCTCGTTTACCGAGATATGTCCGGTGGTATTAACGAAGCATTCTCGGATATC
AsnSerGlyLeuValTyrArgAspMetSerGlyGlyIleAsnGluAlaPheSerAspIle
SalI
GCAGGGGAAGCGGCAGAGTACTTTATGCGTGGCAATGTCGACTGGATTGTCGGCGCGGAT
AlaGlyGluAlaAlaGluTyrPheMetArgGlyAsnValAspTrpIleValGlyAlaAsp
ATTTTTAAATCCTCCGGTGGTCTACGTTATTTCGATCAGCCGTCACGTGATGGCCGCTCG
IlePheLysSerSerGlyGlyLeuArgTyrPheAspGlnProSerArgAspGlyArgSer
ΔH
ATAGATCATGCTTCACAGTATTACAGCGGTATTGATGTTCATTCGAGTGGCGTGTTTAAC
IleAspHisAlaSerGlnTyrTyrSerGlyIleAspValHisSerSerGlyValPheAsn
CGCGCGTTTTACCTACTCGCCAATAAATCGGGTTGGAACGTACGTAAAGGTTTTGAAGTG
ArgAlaPheTyrLeuLeuAlaAsnLysSerGlyTrpAsnValArgLysGlyPheGluVal
TTTGCCGTGGCTAACCAGTTGTACTGGACACCGAACAGCACGTTTGATCAAGGTGGCTGT
PheAlaValAlaAsnGlnLeuTyrTrpThrProAsnSerThrPheAspGlnGlyGlyCys
GGGGTAGTGAAAGCGGCGCAGGATCTCAACTACAACACCGCAGACGTTGTGGCAGCCTTT
GlyValValLysAlaAlaGlnAspLeuAsnTyrAsnThrAlaAspValValAlaAlaPhe
AATACCGTGGGTGTCAATGCTTCTTGTGGCACCACGCCACCACCTGTCGGCAAAGTGCTT
AsnThrValGlyValAsnAlaSerCysGlyThrThrProProProValGlyLysValLeu
GAGAAAGGTAAACCGATCACAGGACTGAGCGGCTCACGTGGAGGAGAAGATTTCTATACC
GluLysGlyLysProIleThrGlyLeuSerGlySerArgGlyGlyGluAspPheTyrThr
TTTACGGTGACCAATTCAGGCAGTGTTGTTGTGTCCATCAGTGGTGGAACGGGCGATGCG
PheThrValThrAsnSerGlySerValValValSerIleSerGlyGlyThrGlyAspAla
GATCTGTATGTCAAAGCGGGCAGCAAACCCACCACCTCTTCTTGGGATTGTCGTCCATAC
AspLeuTyrValLysAlaGlySerLysProThrThrSerSerTrpAspCysArgProTyr
CGTTCAGGCAATGCCGAGCAGTGTTCCATCTCTGCGGTCGTGGGTACGACATACCATGTC
ArgSerGlyAsnAlaGluGlnCysPheIleSerAlaValValGlyThrThrTyrHisVal
ATGTTACGCGGTTACAGTAACTATTCTGGTGTGACGTTACGCTTGGACTAACTTCCTTGC
MetLeuArgGlyTyrSerAsnTyrSerGlyValThrLeuArgLeuAsp***
CACCTACCTGCAACGCCCTCAGCAAAGTCTGAGGGCGTTGTTTTTGAAGGGCAGTTTCTA
GGATGTATCAACTATTTGAGTTGGCTGACCGCCGAAGAAACATTTTCTGCACCTTGGTAA
ATCTGTTCCATGATGGTTGACACTTCCACAATGCGGCCATTGGTTTCATTGGCAATGTGA
GAGACTTGAGAAATAGAGCTGGTTACCGCTTCAGTTAAGGAGAGGTTTTTATTCACCACT
TGATTGATCTCTTCTGTGGCTTTTGAGGTGCGAGAACGCAGTTGACCGACTTCATCGGCA
AACCACCGCAAAACCGCGTCCTTGATCAACCGCTGCGACGCTCTATCGCTGCATTAATGC
GGAGCAGATGGTTTGGTCAGCGATACACTGATGGTTTTGACAATTTCAGAGACATCTTTC
GAGAGAACCACGAGCTGCTCAATCTGTTGTAGTGATTGTTCGATATTGCCCACCATTTTT
TCTGCTAACGACTGAATCTTGCAGTACATGTTCCGCCTTGTGCTACTTGCGAGGTTCTAC
PstI1380
TGAGGTGCTATAGGCGATATTGGCAGCGTCTGTTACTTGCTGTTCACGTAATACCTCTGC
AGTGATGTCTGAGGCGAACTTGACAATTTTATATACCTTATTGTTTTGATCTTTGACCGG
1460
ACTGTAGGAGGCTTGGATCC
BamHI

FIG. 2.


Sal I 520
EcoR I 423
BamH I 417
EcoR I 396
1
4000
500
3500
pUC 19
+ 1.5Kb FRAGMENT
1000
3000
1500
2500
2000
Pst I 1802
BamH I 1876
Sal I 1888
Pst I 1898

INTERNATIONAL SEARCH REPORT

Interna al Application No
PCT/GB 94/01406

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/57 C12N9/52 C12N5/12 C07K14/205 C07K14/28
C07K16/12 C12Q1/68 G01N33/577 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K C12Q G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF BACTERIOLOGY, vol.173, no.11, June 1991, AM. SOC. MICROBIOL., BALTIMORE, US; pages 3311 - 3317 C.C. HASE AND R.A. FINKELSTEIN 'Cloning and nucleotide sequence of the Vibrio cholerae haemagglutinin/protease (HA/protease) gene and construction of an HA/protease-negative strain' cited in the application see page 3312, right column, line 22 - line 24; figure 2 see page 3314, right column, line 11 - line 16 --- -/--	4,5,8, 12,13, 18,22

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
31 October 1994	22 -11- 1994

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Hornig, H

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF BACTERIOLOGY, vol.170, no.9, September 1988, AM. SOC. MICROBIOL., BALTIMORE, US; pages 4309 - 4314 R.A. BEVER AND B.H. IGLEWSKI 'Molecular characterization and nucleotide sequence of the Pseudomonas aeruginosa lastase structural gene' cited in the application see page 4309, right column, line 25 - line 28; figure 2 ---	18,25
X	INFECTION AND IMMUNITY, vol.36, no.3, June 1982, AM. SOC. MICROBIOL., BALTIMORE, US; pages 1199 - 1208 R.A. FINKELSTEIN AND L.F. HANNE 'Purification and characterization of the soluble hemagglutinin (Cholera lectin) produced by Vibrio cholerae' see page 1200, left column, line 57 - right column, line 2 ---	18,25
A	EP,A,0 367 644 (INSTITUT PASTEUR) 9 May 1990 see the whole document ---	1-7, 21-24, 27,28,31
A	INFECTION AND IMMUNITY, vol.60, no.2, February 1992, AM. SOC. MICROBIOL., BALTIMORE, US; pages 472 - 478 R.A. FINKELSTEIN ET AL. 'Vibrio cholerae haemagglutinin/protease, colonial variation, virulence, and detachment' cited in the application see the whole document ---	1-33
P,X	MOLEC. MICROBIOL., vol.13, no.1, 1994, BLACKWELL SCI. PUB., OXFORD, UK; pages 153 - 160 A.W. SMITH ET AL. 'The human gastric pathogen Helicobacter pylori has a gene encoding an enzyme first classified as a mucinase in Vibrio cholerae' see page 154, left column, paragraph 2 - page 158, right column, paragraph 2; figure 2 ---	1-18, 21-25

1 -/--

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	MOLECULAR AND CELLULAR PROBES, vol.7, no.6, December 1993, ACADEMIC PRESS, LONDON, GB; pages 439 - 446 C.R. LOPEZ ET AL. 'Comparison of urease gene primer sequences for PCR-based amplification assay in identifying the gastric pathogen Helicobacter pylori' cited in the application see the whole document	1-7, 21-24

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter ial Application No
PCT/GB 94/01406

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0367644	09-05-90	FR-A-	2637612	13-04-90
		WO-A-	9004030	19-04-90
		JP-T-	3501928	09-05-91